

In Vitro Effects of Mineral Fibers

by Lalita D. Palekar,* Phillip M. Cook† and
David L. Coffin‡

In vivo tests available to determine the toxicity of mineral fibers are too expensive and time-consuming to be regularly employed in the evaluation of the potential health hazard posed by natural and man-made fibers. *In vitro* procedures, while economical, convenient and capable of ranking "relative toxicity," are uncertain predictors of specific lesions. Thus, it is of interest to compare the results of various standard *in vitro* tests with the results of *in vivo* tests.

Data are available for intratracheal and intrapleural exposures of animals to amphibole mineral fibers from UICC amosite and a fibrous form of ferroactinolite. This paper presents data from parallel *in vitro* studies employing these minerals. The methods used were mammalian erythrocyte lysis, Chinese hamster ovary cell clonal cytotoxicity assay, and rabbit alveolar macrophage cytotoxicity assay. The experiments were conducted in triplicate to determine dose effect by mass and by number of fibers with aspect ratios greater than 3. A comparison of relative toxicity was made between the ferroactinolite and amosite. In the erythrocyte system, there was a greater lytic effect per unit of fibers for ferroactinolite than for the UICC amosite.

Introduction

Many data have been collected concerning the reactivity of isolated cells to various mineral fibers and crystals. Tests, such as erythrocyte lysis and toxicity to macrophages, fibroblasts and Chinese hamster ovary (CHO) cells, are frequently used to measure the biological activity of asbestos. Such experiments may provide information about the mechanism of action of mineral fibers against cells and results that will correlate with results of *in vivo* exposures to predict fibrosis or tumorigenesis. If such a correlation can be established, *in vitro* tests will be of great value in determining the toxicity of natural and man-made minerals when evaluation by long-term animal exposure is too expensive and time-consuming. There is considerable evidence (1, 2) that tumorigenesis of both asbestos and certain artificially produced mineral fibers is related to fiber size and geometric configuration. Recent evidence suggests that size relationships may also affect *in vitro* activity (3).

The experiments described here were performed to determine the relative activity of two naturally

occurring minerals (amosite asbestos and ferroactinolite) for three *in vitro* systems on the basis of dose by mass and by number of mineral fibers.

Materials and Methods

Mineral Characterization

Ferroactinolite fibers were extracted from iron-formation rocks by using an air jet mill. The second mineral in the study was UICC amosite asbestos. Both minerals were characterized by using X-ray diffraction electron microscopic methods. The fiber-size and number-concentration analyses were made using a transmission electron microscope. Fiber-size analysis results are given in Table 1; fiber concentration analysis results are presented in Table 2.

The ferroactinolite was composed of silicon, iron, calcium, magnesium, and manganese. The fiber crystal structure was monoclinic amphibole. The mineral particle content was estimated by volume as follows: 50% ferroactinolite fibers, 20% sheet silicates, 5% magnetite particles, 20% ferroactinolite and hornblende fragments (aspect ratio < 3.0), and 5% other minerals.

The UICC amosite was composed of silicon, iron, magnesium, and manganese. The fiber crystal structure was monoclinic amphibole. The mineral particle content was estimated by volume as follows: 92% grunerite fibers, 6% grunerite fragments, and 2% quartz and other minerals.

*Northrop Services, Inc., Research Triangle Park, NC 27709; author to whom correspondence should be addressed.

†U.S. Environmental Protection Agency, Environmental Research Laboratory-Duluth, Duluth, MN 55804.

‡U.S. Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, NC 27709.

Table 1. Fiber dimensions.

Fiber	Parameter	Mean	Median	Range
Ferroactinolite	Length, μm	3.18	1.50	0.3–52.5
	Width, μm	0.41	0.24	0.03–5.23
	Aspect ratio	9.0	6.0	3.0–130.0
Amosite	Length, μm	3.44	1.80	0.15–378.0
	Width, μm	0.29	0.22	0.02–4.08
	Aspect ratio	11.8	7.5	3.0–2825.0

Table 2. Fiber concentration analysis.

Fiber	Number of fibers $\times 10^6/\text{mg}$		Surface area, $\text{m}^2/\text{g} \times 10^6$
	Aspect ratio > 3	Length $> 8\mu\text{m}$ width $< 0.25\mu\text{m}$	
Ferroactinolite	81	0.09	0.8
Amosite	570	8.3	3.0

In Vitro Test Procedures

The three *in vitro* test procedures used in this study were mammalian erythrocyte lysis assay, CHO cell clonal cytotoxicity assay and rabbit alveolar macrophage (RAM) cytotoxicity assay. These procedures are described below.

Mammalian Erythrocyte Lysis Assay. Pooled sheep blood was obtained commercially in Alsever's solution. Just before use, the blood was washed with veronal buffer, and a 2% suspension was prepared for testing. A standard reference of lysate was prepared by mixing the blood suspension with water at a 1:3 ratio. A spectrophotometer was adjusted to read between 0.700 and 0.750 with the lysate at 540 nm.

Known amounts of test minerals were added to test tubes containing 3 mL of veronal buffered saline and incubated for 50 min at 37°C. The tubes were then centrifuged at 365g for 15 min, and the supernatant (lysate) analyzed using the standardized spectrophotometer. Percent hemolysis was determined as follows:

$$\frac{\text{Optical density of sample}}{\text{Optical density of lysate}} \times 100 = \% \text{ Hemolysis}$$

Surface adsorption of hemoglobin was determined to accommodate any error due to the loss of hemoglobin by adsorption. Test samples were added to 4 mL of lysate and incubated for 50 min at 37°C. The tubes were then centrifuged and the suspension was spectrophotometrically analyzed. Percent adsorption was determined as follows:

$$\% \text{ Adsorption} = \{(\text{OD}_s - \text{OD}_l)/\text{OD}_l\} \times 100$$

where OD_s is the optical density of the sample and OD_l is that of the lysate.

If the sample adsorbed hemoglobin, the values of

percent adsorption and hemolysis were added to obtain the actual hemolysis.

Chinese Hamster Ovary Cell Clonal Assay. The CHO cell line was obtained from the American Type Culture Collection. The cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units of penicillin per 100 mL, and 100 μg of streptomycin per 100 mL. The cultures were incubated at 37°C and gassed with 5% CO_2 in air.

For the assays, 25 mm^2 flasks were seeded with 500 cells in 4 mL of nutrient medium and incubated at 37°C for 24 hr to allow cell attachment. After this period, the test substance was added to the flasks and incubated for 6 days. The cultures were then prepared by removing the medium and adding fixative (0.5% NaCl and 4% methanol in 10% formalin). The fixative was then removed, the cells were stained with 0.04% crystal violet, and the colonies were counted. The number of surviving colonies was determined as percent of control (cultures without test particles).

Rabbit Alveolar Macrophage Cytotoxicity Assay. The macrophages were obtained by pulmonary lavage. The rabbits were sacrificed by injecting Nembutal into the marginal ear vein. A tracheotomy was performed and 30 mL of sterile physiological saline was introduced into the trachea. After 15 min, the saline was withdrawn, and the lavage fluid was filtered through sterile gauze into a 250-mL Nalgene centrifuge tube. This procedure was repeated five times. The lavage fluid was pooled, centrifuged at 4°C for 15 min at 365g and decanted. The cell pellet was resuspended in a known amount of physiological saline. The cell viability was determined by light microscopy using the trypan blue exclusion technique (4). The cell number was determined by direct count using a hemocytometer.

The cells were then resuspended in a nutrient medium consisting of Minimum Essential Medium (Eagle) with 20 mM HEPES buffer without L-glutamine, 20% fetal calf serum (heat inactivated), and 4 mg of Gentamycin per 100 mL.

To each flask, 2×10^6 cells were added. The flasks were incubated for 3 hr at 37°C for stabilization, known amounts of test sample were added, and the flasks were incubated for 20 hr (5). The cells were harvested, and viability was determined. To ensure proper comparison, the index of viability was determined as percent of control.

Results

Mammalian Erythrocyte Lysis Assay

When the hemolytic activities of mass doses of ferroactinolite and amosite were considered, amo-

site was determined to be more toxic than ferroactinolite (Fig. 1). The difference in toxicity was greatest at the 5 and 10 mg dose level. At the 20 mg dose, the amosite and ferroactinolite produced the same degree of hemolysis.

In contrast, ferroactinolite was found to be more hemolytic than amosite when the dose was determined as number of fibers (Fig. 2). In general, fewer fibers of ferroactinolite than amosite were required to produce each level of hemolysis. To achieve 60% hemolysis required 1.6×10^9 fibers of amosite. The numbers of fibers estimated to produce various levels of hemolysis are given in Table 3.

Chinese Hamster Ovary Cell Clonal Assay

To evaluate the CHO cytotoxic response, comparisons were made of the results of mass doses of ferroactinolite and UICC amosite. The amosite was found to be much more toxic than the ferroactinolite. At the maximum dose of 1000 $\mu\text{g/mL}$ of ferroactinolite, there were 55% surviving colonies. At the same dose of amosite, there were no surviving colonies. Also, the change in dose response was more gradual for the ferroactinolite than for the amosite (Fig. 3).

Similar response was observed when the dose

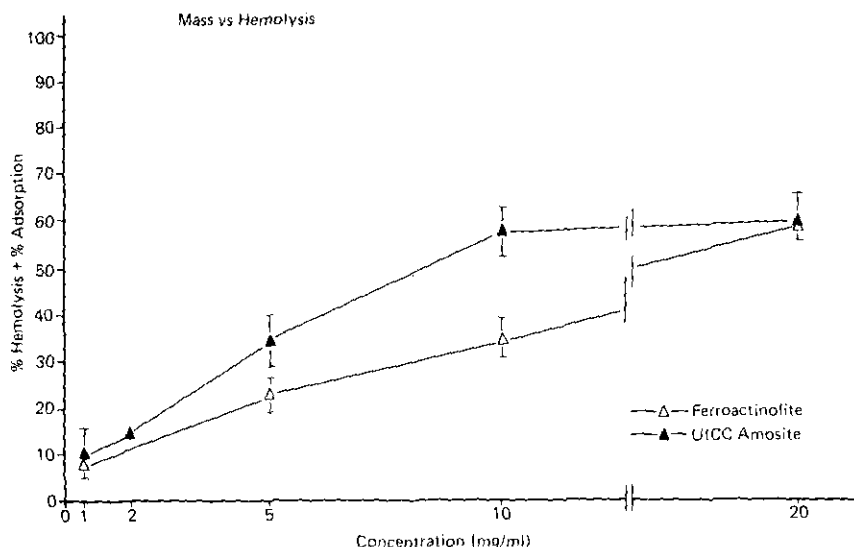


FIGURE 1. Comparison of hemolytic response for equal masses.

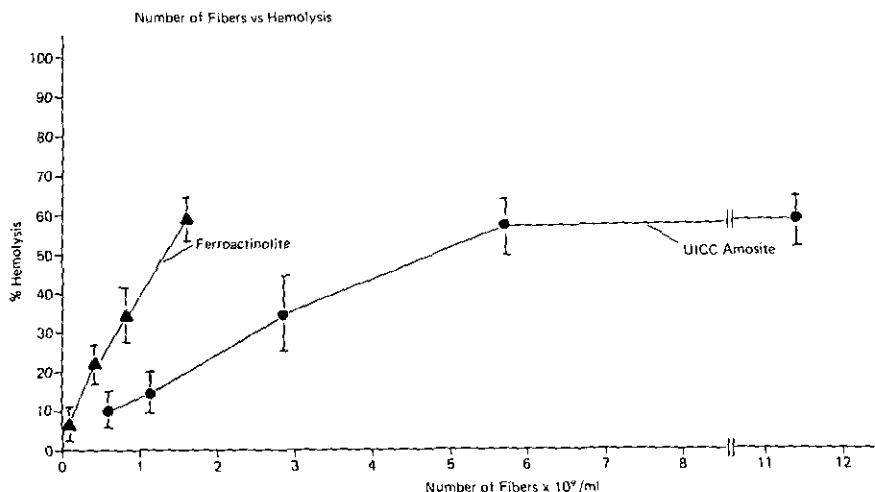


FIGURE 2. Comparison of hemolytic responses for equal numbers of fibers.

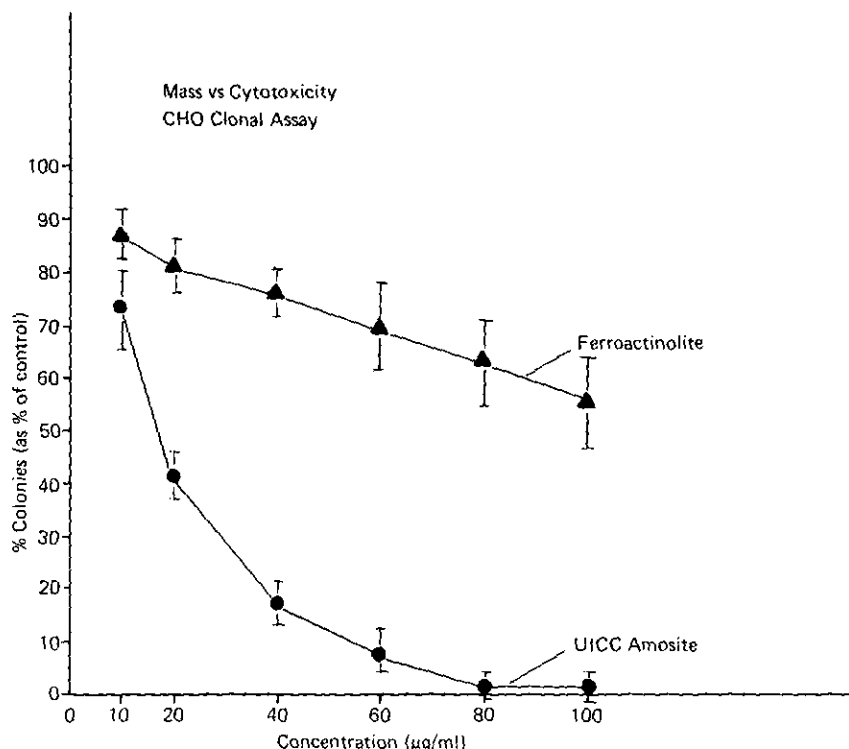


FIGURE 3. Comparison of CHO cytotoxicity response for equal masses.

Table 3. Estimated number of fibers required for hemolysis.

Hemolysis, %	Number of fibers $\times 10^9$	
	Ferroactinolite	Amosite
10	0.15	0.6
20	0.4	1.5
30	0.7	2.5
40	1.0	3.7
50	1.4	4.8
60	1.6	11.0

was measured as number of fibers. More fibers of ferroactinolite were required to achieve the same degree of cytotoxicity (Fig. 4). The numbers of fibers required by the two samples to achieve the same levels of cytotoxicity are given in Table 4.

Rabbit Alveolar Macrophage Assay

Mass doses of UICC amosite were more cytotoxic than the same amounts of ferroactinolite in the RAM assay (Fig. 5). At the highest concentration (1000 µg/mL), the viability for amosite was less than 10%, whereas it was 50% for the ferroactinolite. At the lowest concentration (50 µg/mL), the viability for amosite was 73%, whereas it was 90% for the ferroactinolite.

When the dose was measured as number of fi-

bers, the amosite was again more cytotoxic than the ferroactinolite (Fig. 6). Fewer fibers of amosite were required to achieve the same degree of cytotoxicity. The change in dose response was gradual. The number of fibers required for each level of cytotoxicity is given in Table 5.

Discussion

The two test samples, ferroactinolite and UICC amosite, were tested in three *in vitro* systems (mammalian erythrocyte hemolysis, CHO cell clonal cytotoxicity test, and RAM cytotoxicity assay). Independent variables were dose by mass and dose by number of fibers.

The erythrocyte hemolysis study indicated that, by mass, ferroactinolite was less hemolytic than UICC amosite. In other words, a larger mass of ferroactinolite than amosite was required to produce the same degree of hemolysis. Contrary to this, when number of fibers was considered, ferroactinolite was far more hemolytic than the amosite. In other words, fewer fibers of ferroactinolite than amosite were required to produce the same degree of hemolysis.

The data obtained from the CHO clonal assay for mass dose indicate that ferroactinolite was less tox-

IN VITRO EFFECTS OF MINERAL DUSTS

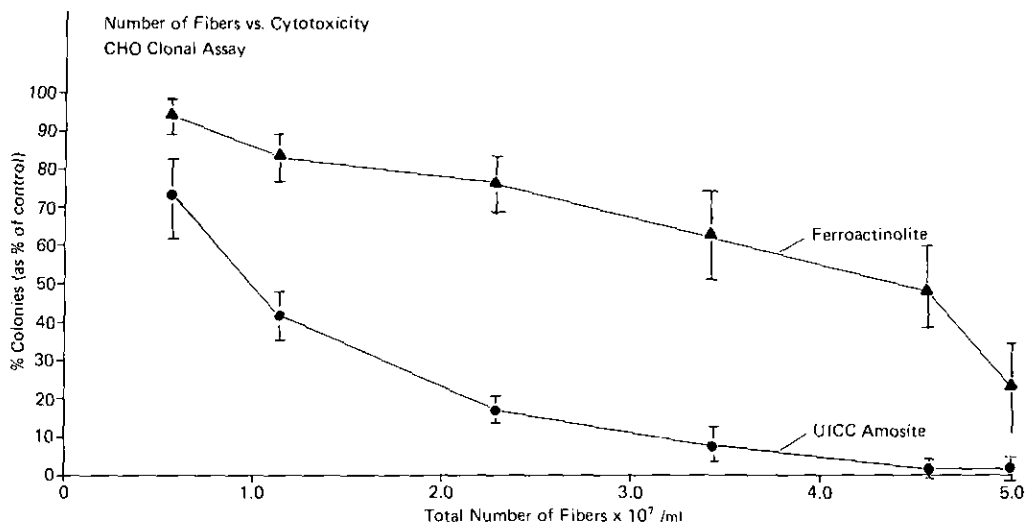


FIGURE 4. Comparison of CHO cytotoxicity responses for equal numbers of fibers.

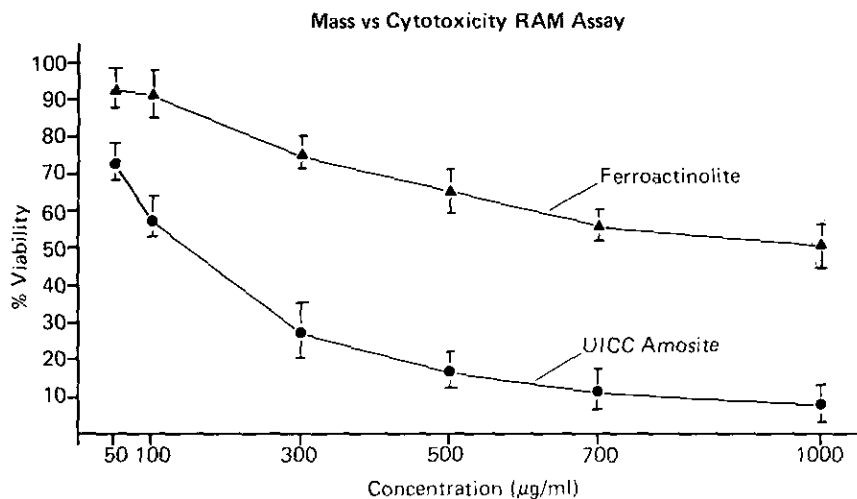


FIGURE 5. Comparison of RAM cytotoxicity response for equal masses.

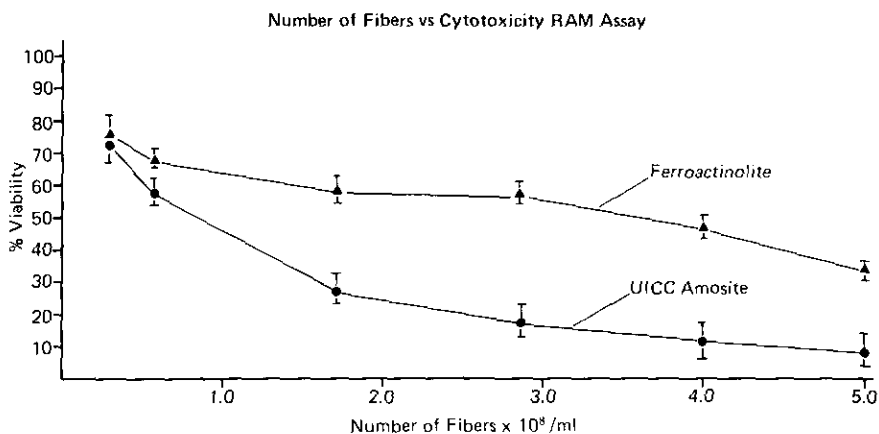


FIGURE 6. Comparison of RAM cytotoxicity response for equal numbers of fibers.

Table 4. Estimated number of fibers required for CHO cytotoxicity.

Colonies, %	Number of fibers $\times 10^7$	
	Ferroactinolite	Amosite
75	2.15	0.58
60	3.55	0.8
50	4.5	1.0
40	4.7	1.2
30	4.85	1.7

Table 5. Estimated number of fibers required for RAM cytotoxicity.

Viability, %	Number of fibers $\times 10^4$	
	Ferroactinolite	Amosite
40	4.4	1.2
50	3.48	0.85
60	1.4	0.5
70	0.5	0.3

ic than UICC amosite, which agreed with the results of the hemolysis study. However, ferroactinolite was again found to be less toxic than amosite when the responses were computed for equal numbers of fibers. This second result conflicts with the results of the hemolysis study.

The third system, RAM cytotoxicity assay, rendered results similar to the CHO clonal assay. Again, ferroactinolite was less cytotoxic than UICC amosite, regardless of whether the responses were compared for equal mass or equal numbers of fibers. The data suggest that the influence of the minerals on erythrocytes is different than that on CHO and RAM cells.

When computing the dose by number of fibers, it was assumed that the sheet silicates and the fragments found in the samples were not lytic or cytotoxic and that the biological activity observed was due only to the fibers. This assumption was based on the findings of Lipkin (4), who showed that the degree of cytotoxicity is dependent on the presence of fibers. Similarly, Palekar et al. (6) demonstrated

that the degree of hemolysis and cytotoxicity depends on the fibrous nature of the minerals.

These *in vitro* data are compared with *in vivo* data in the presentation by Coffin et al. (7). Briefly, the *in vivo* studies showed that the ferroactinolite was equally tumorigenic in the intrapleural studies and more tumorigenic in the intratracheal studies, even when there were fewer ferroactinolite fibers than amosite fibers at the onset of the dose regimen.

The data from the hemolysis assay and the *in vivo* studies show similarity if one looks at the doses that were administered at the onset of the *in vivo* study. The data are similar, in the sense that fewer fibers of ferroactinolite than amosite are required to elicit an equal amount of biological reaction. No correlation has been determined at this time between the data from the other two *in vitro* tests and the *in vivo* studies.

REFERENCES

1. Coffin, D. L., Palekar, L. D., and Cook, P. M. Tumorigenesis by a ferroactinolite mineral. *Toxicol. Letters* 13: 143-150 (1982).
2. Cook, P. M., Palekar, L. D., and Coffin, D. L. Interpretation of the carcinogenicity of amosite asbestos and ferroactinolite on the basis of retained fiber dose and characteristics *in vivo*. *Toxicol. Letters* 13: 151-158 (1982).
3. Stanton, M. F., Layard, M., Tegeris, A., Miller, E., May, M., and Kent, E. Carcinogenicity of fibrous glass: pleural response in the rat in relation to fiber dimension. *J. Natl. Cancer Inst.* 58: 587-603 (1977).
4. Lipkin, L. E. Cellular effects of asbestos and other fibers: Correlations with *in vivo* induction of pleural sarcoma. *Environ. Health Perspect.* 34: 91-102 (1980).
5. Hanks, J. H., and Wallace, J. H. Determination of cell viability. *Proc. Soc. Exptl. Biol. Med.* 98: 188-192 (1958).
6. Palekar, L. D., Spooner, C. M., and Coffin, D. L. Influence of crystallization habit of minerals on *in vitro* cytotoxicity. *Ann. N.Y. Acad. Sci.* 330: 673-686 (1979).
7. Coffin, D. L., Palekar, L. D., and Cook, P. M. Correlation of *in vitro* and *in vivo* methods by means of mass dose and fiber distribution for amosite and fibrous ferroactinolite. *Environ. Health Perspect.* 51: 49-53 (1983).